THE INFLUENCE OF MOLYBDENUM AND VANADIUM ON NITROGEN FIXATION BY *CLOSTRIDIUM BUTYRICUM* AND RELATED ORGANISMS.

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(Plate vii.)

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Introduction.

The fixation of elementary nitrogen by *Azotobacter* is strongly accelerated by small quantities of molybdenum, as shown by Bortels (1930, 1936), Birch-Hirschfeld (1932), Kluyver and van Reenen (1933), Burk and Horner (1935), and Horner *et al.* (1942). By means of a manometric technique, Burk and Horner (1935) were able to detect a stimulating effect of molybdenum in a concentration as minute as 10^{-11} molar, or about 0·001 microgram per litre of medium. Molybdenum acts as a specific catalyst and can be replaced only by vanadium which at optimal concentration shows an effect of about one-half to two-thirds of that of molybdenum (Bortels, 1933, 1936; Burk and Horner, 1935; Horner *et al.*, 1942). Burk and Horner (1935) tested 22 other elements, mostly heavy metals, with negative results, and Bortels (1936) more than 40 (not specified). A stimulating effect of tungsten has sometimes been reported, but this was shown by Horner *et al.* (1942) to be spurious and due to the difficulty of separating tungsten and molybdenum completely. Earlier findings of stimulation of *Azotobacter* by other elements, such as manganese, titanium, thorium, uranium, etc., may probably be reduced to a similar cause.

The evidence is somewhat less clear-cut as regards the assimilation of combined nitrogen by *Azotobacter*. Birch-Hirschfeld (1932) found no influence of molybdenum on growth with nitrate. Burk and Horner (1935) made the same statement, but found a small acceleration of growth with ammonia, and a still smaller one with urea. Bortels (1936), on the other hand, found that molybdenum promoted the uptake of nitrate and permitted the fixation of nitrogen in the presence of nitrate, ammonia and asparagin. Horner and Allison (1944) confirmed this for nitrate and asparagin. Results found by Wilson *et al.* (1943) suggest that this discrepancy may be explained through previous adaptation of *Azotobacter* to nitrate.

There seems to be only one positive observation on record regarding the influence of molybdenum on the anaerobic nitrogen-fixing bacteria: *Clostridium butyricum* and related species. Bortels (1936) found in a single experiment with a pure culture of these organisms that the gains of nitrogen were increased some 2 to 2½ times by small doses of molybdenum and vanadium. Much earlier, Krzemieniewski (1908) had stated that humic acid stimulates nitrogen fixation by butyric acid bacilli as well as *Azotobacter*, in which the effect of humic acid has later been found to be chiefly due to molybdenum. Since there is some evidence that the clostridia may, by virtue of their wider distribution and usually greater abundance in natural habitats, equal or exceed the more efficient but less numerous *Azotobacter* as agents of biological nitrogen fixation, we have subjected this problem to a more detailed investigation.

The nutrition and especially the nitrogen fixation process of *Cl. butyricum* present several obscure aspects. Unlike *Azotobacter*, which can synthesize all its organic growth-factors, the clostridia demand certain preformed growth-compounds. No systematic study of the requirements of *Cl. butyricum* appears to have been undertaken.

For the closely related *Cl. acetobutylicum*, of which some strains are also able to fix nitrogen (McCoy *et al.*, 1928), the essential accessory factors for growth in a synthetic medium with ammonia- or amino-nitrogen have been shown by Oxford *et al.* (1940), Rubbo *et al.* (1941) and Lampen and Peterson (1943) to be biotin and *p*-aminobenzoic acid. Lampen and Peterson found a strain of *Cl. butyricum* that grew well with biotin as the only growth-compound. Woolley *et al.* (1939) studied an unidentified growth-factor, probably a mixture of biotin and *p*-aminobenzoic acid, which supported growth of *Cl. acetobutylicum* and two strains of *Cl. butyricum*, but not of a third strain of the *pasteurianum*-type, probably identical with the one received by us (cf., below).

The need of nitrogen-fixing clostridia for organic growth-compounds thus appears clearly established, but the medium for nitrogen fixation experiments has nearly always been some modification of the synthetic and, if pure, growth-compound-free glucose solution used by Winogradsky (1895). Growth in such a medium may occur only if a sufficient amount of growth-compounds is carried over with the inoculum, and the size of this is rarely stated. Bredemann (1909*b*), for instance, used 10 ml. of culture fluid per litre of medium, and McCoy *et al.* (1928) state that as inocula they used "measured amounts of 24 hour corn mash cultures"; the amounts were not specified, and the authors make the significant remark that inocula from previous cultures in Winogradsky's or other synthetic media were weak and uncertain. An inoculum large enough to contain sufficient growth-compounds for the subculture would be quite likely also to provide an adequate supply of molybdenum. This was apparently not the case in the experiment of Bortels (1936), who states that the mother-culture was grown in molybdenum-free medium, but it may explain the negative result of an earlier tentative experiment by one of us (Jensen, 1941), in which it was found necessary to use an inoculum of several drops of glucose broth culture per 50 ml. solution in order to start growth.

The quantity of nitrogen fixed by *Cl. butyricum*, especially the *pasteurianum*-type, has usually been found to vary between 1 and 3 mgm. per gm. fermented sugar (Winogradsky, 1895; Pringsheim, 1908; Bredemann, 1909*b*; Lantsch, 1921; McCoy *et al.*, 1928; Willis, 1934; Sjolander and McCoy, 1937). Higher yields have rarely been observed. Bredemann (1909*b*) found occasional gains up to 6 mgm.; McCoy *et al.* (1928) showed that the slowly-fermenting strains of the *saccharobutyricum*-type could fix from 4·3 to 7 mgm. nitrogen per gm. sugar, and Kostytchev (1924) mentions a yield of 12·5 mgm. In associated cultures of clostridia and cellulose-decomposing bacteria the yield of fixed nitrogen has repeatedly been found to reach 7 to 10 mgm. per gm. cellulose lost (Pringsheim, 1910; Jensen, 1941).

It is probable that the strongest nitrogen fixation may occur if the medium contains adequate supplies of organic growth-compounds and of molybdenum but a minimum of available nitrogen which tends to inhibit fixation, as shown by Winogradsky (1895) and Omeliansky (1916). Therefore, we have endeavoured to develop a medium deficient in molybdenum and nitrogen while providing the organic growth-factors needed by *Cl. butyricum*.

Experimental.

Methods and Organisms.—Eight freshly isolated strains of *Cl. butyricum* were tested; four of these (Nos. 1, 3, 6*a* and 6*b*) were isolated from soil, three (Nos. 7*a*, 7*b* and 8) from garden compost, and one (No. 5) from flax straw. Enrichment cultures were made by inoculation of Winogradsky's solution with soil or compost suspension or liquid from retting flax, and incubation at 30°C. after pasteurization for 5 to 10 minutes at 80°C. When gas evolution appeared and typical clostridia were seen by microscopic examination (sometimes after two or three transfers in the same medium), subcultures were made in potato-medium (test-tubes with 12–15 ml. tap water, a two-inch deep layer of small pieces of peeled potatoes, and some calcium carbonate). These cultures always showed vigorous fermentation, smelt of butyric acid, and showed an abundance of clostridia staining blue with iodine after 24 hours at 30° or 37°C. Pasteurization was then repeated, and plate cultures were made on glucose agar with

lucerne-root-extract and incubated in hydrogen atmosphere in an anaerobic jar at 30°C. Isolated colonies of microscopically pure appearance were transferred to tubes of potato medium which supported good growth of the pure cultures in atmospheric air if the medium was boiled and cooled immediately before inoculation. The same medium, sometimes with addition of a small quantity of soil, was used for maintenance of the cultures. The purity of these was tested by cultivation aerobically in nutrient broth, in glucose-peptone solution, and on plates of nutrient agar; slopes of fresh glucose agar often showed growth of the clostridia in the condensation-water, as also observed by Bredemann (1909*b*).

Besides these fresh isolates we have tested a strain of the *pasteurianum*-type (originally isolated by Winogradsky) from the Department of Agricultural Bacteriology, University of Madison, U.S.A., and a strain of *Cl. acetobutylicum* from the Department of Bacteriology, University of Melbourne. These two strains are in the following tables called "W" and "B", respectively.

Morphologically, all strains showed the typical appearance: big rods with rounded ends, mostly single or in pairs, rarely in chains of 3 or 4 cells, actively motile, Gram-positive when young but rapidly becoming Gram-negative, and developing into typical spindle-shaped clostridia that stain blue with iodine and contain oval, subterminal endospores. The vegetative cells measured 0·5–1·2 × 2–8μ, sometimes up to 10μ long and 2·5μ thick in the clostridium-stage, and the endospores 1·0–1·2 × 1·5–1·8μ. In some strains, for instance, Nos. 1 and 3, the cells were predominantly long and slender, in No. 5 short and thick, almost lemon-shaped in the clostridium-stage.

The fermentation reactions of the strains were tested towards some of the compounds most likely to distinguish between the different types of butyric acid bacilli according to McCoy *et al.* (1930). The medium was Winogradsky's solution (see below) with 0·2% ammonium lactate, 0·2% potato-extract-concentrate, and 1·0% carbon compound, but no calcium carbonate. Test-tube cultures incubated four days at 35°C. in hydrogen atmosphere showed the following reactions:

Carbon Compound.	Fermentation (Acid and Gas) by Strain No.									
	1	3	5	6*a*	6*b*	7*a*	7*b*	8	W	B
Mannite	+	−	?	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	−	−	+	−	+
Starch	+	−	+	+	+	−	−	+	−	+

Strains 1, 5, 6*a*, 6*b* and 8 appear to represent the starch-fermenting *saccharobutyricum*-type and strains 7*a* and 7*b* the *pasteurianum*-type, of which strain W is the prototype, while strain 3 seems to represent an atypical form (McCoy *et al.* (1930) found only one strain among 35 unable to ferment mannite. In another series of investigations, Sjolander and McCoy (1937) found 5 out of 20 strains unable to ferment mannite, but these all fermented starch.) It also differs from the others in causing a vigorous reduction of nitrate (0·2% $NaNO_3$ in Winogradsky's solution, incubated in hydrogen atmosphere) to ammonia with formation of small amounts of nitrite, probably as an intermediate product.

The basal medium for the nitrogen-fixation experiments was a modification of Winogradsky's solution: glucose 20·0 gm., K_2HPO_4 0·5 gm., KH_2PO_4 (sometimes omitted) 0·5 gm., $MgSO_4$ 0·1 gm., NaCl 0·1 gm., $FeSO_4$ 0·01 gm., $MnSO_4$ 0·01 gm. (this was found unnecessary and was often omitted), $CaCO_3$ 4·0 or 5·0 gm., distilled water 1,000 ml. The amount of calcium carbonate was reduced from 30 gm., as used by Winogradsky and most others, in order to minimize the amounts of molybdenum and other trace element impurities that might be introduced with it. The chemicals were of ordinary analytical purity.

Some difficulty was at first experienced in obtaining growth with small inocula. Even in solution with combined nitrogen, biotin and *p*-aminobenzoic acid (the medium

of Lampen and Peterson, 1943), growth from an inoculum of 0·01 ml. potato-medium culture per 10 ml. solution took place only irregularly and after a long lag period. In this connection it is noteworthy that Rubbo *et al.* (1941) and Lampen and Peterson (1943) used inocula of about 0·1 ml. per 5 or 10 ml. of medium, and that Oxford *et al.* (1940) found the growth of *Cl. acetobutylicum* in synthetic solution with biotin and an unknown growth-factor (probably *p*-aminobenzoic acid) accelerated by small additional amounts of cystein, valin and methionin. On the other hand, prompt and vigorous growth within 24 hours at 37°C. was obtained when potato extract was added to the medium, and the same effect was shown by a potato-extract-concentrate from which most of the nitrogenous and mineral constituents had been removed, and which was prepared as follows:

Five hundred gm. of peeled and finely-chopped potatoes were heated for 3 to 4 hours in the steamer with one litre of tap water; the liquid was strained off, and the residue was washed with hot water to make one litre of extract, which was filtered on the Buchner funnel to remove the suspended solid matter. The clear extract was concentrated to about 50 ml. by evaporation on the water-bath, 600 ml. ethyl alcohol were added, the mixture was allowed to stand for at least 24 hours, and the voluminous precipitate was removed by filtration. The alcohol was now distilled off and the extract was finally brought to a volume of 50 ml., sterilized by autoclaving, and stored at refrigerator temperature. Two batches of extract were used in the course of the work, in the following called G.F.1 and G.F.2; the second had a somewhat higher nitrogen and growth-factor content than the first.

For the nitrogen-fixation experiments, 0·2 to 0·5% G.F.-solution was added to the glucose solution, together with one γ of pure biotin (from Lilley & Co.) per litre, and a similar quantity of *p*-aminobenzoic acid. This basal medium was highly deficient in available molybdenum and vanadium, as shown by several tests with a vigorously nitrogen-fixing strain of *Azotobacter chroococcum*. Table 1 gives the results; especially G.F.2 appears almost devoid of molybdenum and vanadium, which seem to be carried down almost quantitatively in the alcohol-precipitate.

TABLE 1.

Response of Azotobacter chroococcum *to Molybdenum and Vanadium added to Winogradsky's Solution.*

Exp. No.	Medium and Incubation.	Mgm. N per Culture. *a.*	*b.*	Gain of N, mgm. *a.*	*b.*
I	40 ml. solution, 0·2% G.F.1. Initial N-content, 0·48 mgm. Inc. 7 d. 30° C.				
	Cultures in: Basal medium (control)	1·59– 1·63		1·11– 1·15	
	Do. +10 p.p.m. Na_2MoO_4	11·57–11·95		11·09–11·47	
	Do. +10 ,, NH_4VO_3	8·38– 8·46		7·90– 7·98	
II	25 ml. solution, 0·5% G.F.1. Initial N-content, 0·32 mgm. Inc. 8 d. 30° C.				
	Cultures in: Basal medium (control)	1·11– 1·53		0·79– 1·21	
	Do. +1·4 p.p.m. $VOSO_4$	3·91– 4·10		3·59– 3·78	
III	25 ml. solution, 0·4% G.F.2. Initial N-content, 0·70 mgm. Inc. 7 d. 30° C.				
	Cultures in: Basal medium (control)	0·84– 0·85		0·14–0·15	
	Do. +10 p.p.m. $VOSO_4$	4·30		3·60	
IV	20 ml. solution with and without 0·2% G.F.2. Inc. 10d. 22–25° C. Initial N-content, −G.F., 0·15 mgm., +G.F., 0·44 mgm.				
	Cultures in: Basal medium −G.F.	0·26– 0·29		0·11– 0·14	
	Do. +4·6 p.p.m. Mo	2·73		2·58	
	Basal medium +G.F.	0·52– 0·56		0·08– 0·12	
	Do. +0·002 p.p.m. Mo	1·67		1·23	
	Do. +4·6 ,, Mo	2·87		2·43	

The medium was used in portions of 50 ml. in 100-c.c. round flasks or Erlenmeyer flasks of Pyrex glass, which before use were cleaned with a hot solution of potassium bichromate and sulphuric acid, thoroughly washed, and boiled in distilled water; inconsistent results with the molybdenum-free basal medium were sometimes found if this cleansing was omitted. Molybdenum was added as sodium molybdate, vanadium as ammonium vanadate (NH_4VO_3) or vanadyl sulphate ($VOSO_4$). The inoculum consisted of one or two drops of a vigorously fermenting 24-hours culture in potato-medium. Growth usually appeared within two days, whereas smaller inocula either showed a long lag period or failed to grow altogether. Very little available molybdenum and vanadium was carried over with the inoculum, as shown by an assay with *Azotobacter*:

Duplicate 25-ml. portions of Winogradsky's solution, without potato-extract, and with sucrose instead of glucose, were given additions of 2, 5 and 10 drops of potato-culture of strain W, sterilized, inoculated with *Az. chroococcum*, and incubated 10 days at 30–32°C. Nitrogen determinations showed the following results:

Addition to Medium.	Nitrogen Fixed. Mgm.	
	a	*b*
Nil (control)	0·28	0·34
2 drops of *Clostridium*-culture	0·28	0·43
5 „ „ „ „	0·30	0·34
10 „ „ „ „	0·36	0·37
10 p.p.m. Na_2MoO_4 (0·12 mgm. Mo per culture)	4·20	4·80

The cultures of clostridia were incubated in big vacuum desiccators or anaerobic jars with an atmosphere of nitrogen gas that had first been passed through alkaline pyrogallol. The temperature of incubation was normally 30°C.; in a few instances, fluctuations in the temperature of the incubation-room caused rises to 32–35°C., which did not appear to make any difference to the results. Duplicate flasks analysed immediately after inoculation served as controls showing the initial nitrogen-content. Uninoculated flasks sometimes showed a small but definite loss of nitrogen during incubation (cf., Löhnis, 1930), and also occasional cultures that failed to grow (Table 5, II–III); therefore the initial nitrogen-content was subtracted from that of the incubated cultures to give the net gain of nitrogen. This was determined by the Kjeldahl method, with selenium as a catalyst; N/28 sulphuric acid and sodium hydroxide, with methyl red and methylen blue as indicator, were used for the titration. In some cases an aliquot of the culture was taken for determination of residual glucose by the method of Lane and Eynon, adapted for small quantities by Cole. All subsequent data, unless otherwise stated, refer to 50 ml. of medium (1 gm. of glucose).

The Effect of Molybdenum and Vanadium on Nitrogen Fixation by Various Strains.— The data collected in Table 2 show that all the strains are able to fix small quantities of nitrogen in the basal medium, but the gains are strongly increased, mostly three- to sixfold, by the addition of 5 or 10 parts per million of sodium molybdate. The upper and lower limits of fixation by the nine strains of *Cl. butyricum* are:

	−Mo	+Mo
Lowest gain, mgm. N	0·08	1·53
Highest „ „ „	0·96	3·95
Mean „ „ „	0·48	2·70

Cl. acteobutylicum fixes rather less nitrogen, as also found by McCoy *et al.* (1928), but the stimulating effect of molybdenum is very marked.

Vanadium, either as sulphate or as vanadate, has no influence on strains 1, 3, 5, 8 (probably), and *Cl. acetobutylicum*, but gives considerable stimulation of the others. In strains 6*a* and 6*b* it even appears equal to molybdenum, while in 7*a*, 7*b* and W its effect is about one-half to two-thirds of that of molybdenum, as in *Azotobacter*. The beneficial effect of the two elements became apparent at an early stage of growth, by increased gas formation and turbidity. Plate vii shows the appearance of some typical cultures. The results thus confirm the finding of Bortels (1936), except that not all strains respond to vanadium.

TABLE 2.

Nitrogen Fixation by Various Strains of Cl. butyricum *and* Cl. acetobutylicum.

Exp. No.	Medium and Incubation.	Mgm. N per Culture. *a.*	*b.*	Gain of N, mgm. *a.*	*b.*
I	*Strain* 1.—0·5% G.F.1. Inc. 14 d. 30° C. Initial N-content, 0·80 mgm.				
	Cultures in: Basal medium (control)	0·88–0·92		0·08–0·12	
	Do. +10 p.p.m. Na_2MoO_4	3·31–3·99		2·51–3·19	
II	*Strain* 3.—As (1).				
	Cultures in: Basal medium (control)	0·88–0·89		0·08–0·09	
	Do. +10 p.p.m. Na_2MoO_4	3·70–3·88		2·90–3·08	
III	*Strain* 3.—0·2% G.F.2. Inc. 11 d. 30° C. Initial N-content, 0·96 mgm.				
	Cultures in: Basal medium (control)	1·13–1·23		0·17–0·27	
	Do. +5 p.p.m. Na_2MoO_4	3·02–3·04		2·06–2·08	
	Do. +5 ,, $VOSO_4$	1·15–1·23		0·19–0·27	
IV	*Strain* 1.—0·4% G.F.1. Inc. 14 d. 30° C. Initial N-content, 0·64 mgm.				
	Cultures in: Basal medium (control)	0·97–1·03		0·33–0·39	
	Do. +10 p.p.m. Na_2MoO_4	3·84		3·20	
	Do. +1·5 ,, $VOSO_4$	1·12		0·48	
	Do. +10 ,, Na_2WO_4*	2·98–3·56		2·34–2·92	
V	*Strain* 1.—0·4% G.F.2. Inc. 14 d. 30° C. Initial N-content, 1·72 mgm.				
	Cultures in: Basal medium (control)	1·95–1·95		0·23–0·23	
	Do. +10 p.p.m. Na_2MoO_4	3·36–3·40		1·64–1·68	
	Do. +4 ,, NH_4VO_3	2·04–2·05		0·32–0·33	
VI	*Strain* 5.—0·2% G.F.1. Inc. 14 d. 37° C. Initial N-content, 0·54 mgm.				
	Cultures in: Basal medium (control)	1·33–1·50		0·79–0·96	
	Do. +10 p.p.m. Na_2MoO_4	3·53–4·49		2·99–3·95	
	Do. +10 ,, $VOSO_4$	1·23–1·28		0·69–0·74	
VII	*Strain* 5.—0·4% G.F.2. Inc. 14 d. 30° C. Initial N-content, 1·72 mgm.				
	Cultures in: Basal medium (control)	1·95–2·04		0·23–0·32	
	Do. +10 p.p.m. Na_2MoO_4	3·25–3·63		1·53–1·91	
	Do. + 4 ,, NH_4VO_3	1·94–2·48		0·22–0·76	
VIII	*Strain* 6*a*.—0·4% G.F.1. Inc. 14 d. 30° C. Initial N-content, 0·75 mgm.				
	Cultures in: Basal medium (control)	1·17–1·38		0·42–0·63	
	Do. +10 p.p.m. Na_2MoO_4	2·55–2·73		1·80–1·98	
	Do. +10 ,, $VOSO_4$	2·50–3·16		1·75–2·41	
IX	*Strain* 6*b*.—As VIII.				
	Cultures in: Basal medium (control)	1·27–1·30		0·52–0·55	
	Do. +10 p.p.m. Na_2MoO_4	2·52–3·25		1·77–2·50	
	Do. +10 ,, $VOSO_4$	2·61–2·74		1·86–1·99	
X	*Strain* 7*a*.—0·2% G.F.2. Inc. 12 d. 30° C. Initial N-content, 0·96 mgm.				
	Cultures in: Basa lmedium (control)	1·32–1·44		0·36–0·48	
	Do. + 5 p.p.m. Na_2MoO_4	4·02–4·46		3·06–3·50	
	Do. + 5 ,, $VOSO_4$	3·09–3·17		2·13–2·21	
XI	*Strain* 7*b*.—As X.				
	Cultures in: Basal medium (control)	1·50–1·58		0·54–0·62	
	Do. + 5 p.p.m. Na_2MoO_4	3·84–4·49		2·98–3·53	
	Do. + 5 ,, $VOSO_4$	2·60–2·90		1·64–1·94	

* *Cf.* Table 5.

TABLE 2.—*Continued.*

Nitrogen Fixation by Various Strains of Cl. butyricum *and* Cl. acetobutylicum.

Exp. No.	Medium and Incubation.	Mgm. N per Culture. *a.* *b.*	Gain of N, mgm. *a.* *b.*
XII	*Strain* 8.—As X and XI.		
	Cultures in Basal medium (control)	1·50–(lost)	0·54
	Do. + 5 p.p.m. Na_2MoO_4	3·61–3·63	2·65–2·67
	Do. + 5 „ $VOSO_4$	1·51–2·13	0·55–1·17
XIII	*Strain W.*—0·4% G.F.1. Inc. 14 d. 30° C. Initial N-content, 0·64 mgm.		
	Cultures in: Basal medium (control)	1·19–1·39	0·55–0·75
	Do. +10 p.p.m. Na_2MoO_4	4·31–4·44	3·67–3·80
	Do. + 4 „ NH_4VO_3	2·89–3·19	2·25–2·55
XIV	*Strain W.*—0·4% G.F.2. Inc. 12 d. 30° C. Initial N-content, 1·72 mgm.		
	Cultures in: Basal medium (control)	2·47–2·57	0·75–0·85
	Do. +10 p.p.m. Na_2MoO_4	5·30–(>4·30)*	3·58–(>2·58)
	Do. + 5 „ VOSO	3·32–3·42	1·60–1·70
XV	*Cl. acetobutylicum* (*B*). 0·2% G.F.2. Inc. 12 d. 32–35° C. Initial N-content, 1·05 mgm.		
	Cultures in: Basal medium (control)	1·25–1·30	0·20–0·25
	Do. +10 p.p.m. Na_2MoO_4	2·53–2·64	1·48–1·59
	Do. +10 „ $VOSO_4$	1·26–1·28	0·21–0·23

* Some of the ammonia was lost during the distillation.

A few additional tests were made with a strain of *Aerobacillus polymyxa*, usually regarded as identical with *Bac. asterosporus*, which Bredemann (1909*a*) found able to fix nitrogen. No fixation could be detected in our cultures, either aerobically or in nitrogen-atmosphere, although some growth took place. It is possible that nitrogen-fixing power might be restored by soil passage as found by Bredemann.

Residual glucose was determined in some of the cultures and the gains of nitrogen calculated per gm. of sugar that had disappeared. These results are shown in Table 3, and agree with the current statement that the clostridia mostly fix from one to three mgm. nitrogen per gm. consumed sugar; indeed the higher of these values is only reached or exceeded where molybdenum is added. The same is the case in several other experiments (Table 2, VI, X, XI, XIII, XIV; Table 4, III), where the fixation ranges between 3 and 4 mgm. per 50 ml. with 1 gm. glucose.

It is possible, however, that the yields of nitrogen are really higher than they appear, particularly in the molybdenum-deficient media, since some of the consumed sugar is probably not used for nitrogen fixation but for growth with the combined nitrogen that the potato-extract contains. It was shown that this nitrogen is available for growth and fermentation, by cultivating two strains for 12 days at 30°C. in a hydrogen atmosphere and in a solution containing 1·0% glucose, 0·2% G.F.2, and 10 p.p.m. sodium molybdate. The result was as follows:

	Strain 1.		Strain 3.	
	−Mo	+Mo	−Mo	+Mo
Mgm. glucose consumed per 50 ml. medium—				
a	..144	158	158	290
b	..146	164	171	300

It is noteworthy that the sugar consumption by one of the strains is nearly doubled by the addition of molybdenum, although, of course, no nitrogen was fixed (initial nitrogen-content 1·00 mgm., after incubation 0·96–1·10 mgm.). A correction for this effect of the combined nitrogen would obviously make the economy of nitrogen fixation appear higher, especially in the absence of molybdenum, but it is uncertain whether

TABLE 3.

Consumption of Glucose and Fixation of Nitrogen per Gram of Glucose consumed. (*Cf.*, *Table* 2.)

Strain and Exp. No.	Control.		+Mo.		+V.	
	Glucose Consumed, mgm.	N Fixed, mgm.	Glucose Consumed, mgm.	N Fixed, mgm.	Glucose Consumed, mgm.	N Fixed, mgm.
Strain 1, Exp. I—						
a.	170	0·5	649	3·9	—	—
b.	146	0·8	627	5·1	—	—
Strain 3, Exp. II—						
a.	170	0·5	676	4·3	—	—
b.	140	0·6	710	4·3	—	—
Strain 3, Exp. III—						
a.	148	1·1	742	2·8	176	1·1
b.	170	1·6	718	2·9	198	1·4
Strain 7*a*, Exp. X—						
a.	323	1·1	997	3·1	997	2·2
b.	509	0·9	997	3·5	997	2·2
Strain 7*b*, Exp. XI—						
a.	281	1·9	997	3·0	997	1·6
b.	313	2·0	997	3·5	997	1·9
Strain 8, Exp. XII—						
a.	227	2·4	997	2·7	315	1·7
b.	—	—	997	2·7	494	2·4
Cl. acetobutylicum, Exp. XV—						
a.	334	0·6	815	2·0	378	0·6
b.	365	0·6	840	1·8	362	0·6

the amount of sugar used up in this way is the same in a hydrogen atmosphere and where nitrogen gas is available for fixation.

Winogradsky (1895), Pringsheim (1908) and Omeliansky (1916) observed that the yield of nitrogen fixed per gm. fermented sugar increased with decreasing glucose concentration of the medium. A test was performed with strain W in order to ascertain if this also applies when an adequate supply of molybdenum is given. The medium contained 0·4% G.F.2, 10 p.p.m. sodium molybdate, and four concentrations of glucose, and had an initial nitrogen content of 1·40 mgm. The following results were found after incubation for 14 days at 32–35°C.:

Per cent. glucose in medium	0·5	1·0	2·0	3·0
Gain of nitrogen, mgm.—				
a	1·52	2·37	3·53	3·93
b	1·48	2·22	3·43	3·99
Consumption of glucose, mgm.—				
a	278	480	926	1,069
b	278	480	926	1,052
Mgm. nitrogen fixed per gm. glucose consumed—				
a	5·5	4·9	3·8	3·7
b	5·3	4·6	3·7	3·8

There is a clear indication that the lower concentrations of sugar are used more economically. The apparent difference is not very great, but here again some glucose has doubtless been used at the expense of the combined nitrogen, and since the amount of this was constant, a correction would particularly increase the yields of nitrogen with the lower sugar concentrations. Phenomena of this kind might explain the high

economy of nitrogen fixation in associated cultures of clostridia and cellulose-decomposing bacteria, where the concentration of sugar produced from the cellulose is kept at a low level by the clostridia and the available nitrogen-compounds removed by the cellulose-decomposers.

The Effective Range of Concentration of Molybdenum and Vanadium.—In the previous experiments the concentrations of molybdenum corresponded to 2·33–4·66 p.p.m., and those of vanadium to 0·47–3·13 p.p.m. These amounts are very large in comparison with those sufficient for *Azotobacter*, and the effect of varying concentrations was tested next. A preliminary experiment with strain 3 showed practically the same gains of nitrogen at concentrations of molybdenum ranging from 0·01 to 5·0 p.p.m., but this experiment was vitiated by an abnormally strong fixation in one of the control cultures. The results of the main experiments are seen in Table 4.

TABLE 4.
Effective Range of Concentration of Molybdenum and Vanadium.

Exp. No.	Strain, Medium and Incubation.	Mgm. N per Culture. *a.* *b.*	Gain of N, mgm. *a.* *b.*
I	*Strain* 3.—0·4% G.F.1. Initial N-content, 0·84 mgm. Inc. 14 d. 30–32° C.		
	Cultures in: Basal medium (control)	1·31–1·34	0·47–0·50
	Do. +0·00002 p.p.m. Mo	0·97–1·15	0·13–0·31
	Do. +0·0001 ,, Mo	1·20–1·23	0·36–0·39
	Do. +0·0005 ,, Mo	1·49–1·75	0·65–0·91
	Do. +0·002 ,, Mo	1·75–2·05	0·91–1·21
	Do. +0·01 ,, Mo	3·18–3·20	2·34–2·36
II	*Strain W.*—0·25% G.F.2. Initial N-content, 1·47 mgm. Inc. 14 d. 30–35° C.		
	Cultures in: Basal medium (control)	2·00–2·03	0·56–0·67
	Do. +0·0001 p.p.m. Mo	1·75–2·48	0·28–1·01
	Do. +0·0005 ,, Mo	2·05–2·25	0·58–0·78
	Do. +0·002 ,, Mo	2·61–2·66	1·14–1·19
	Do. +0·01 ,, Mo	3·65–4·15	2·18–2·68
	Do. +0·1 ,, Mo	4·23–4·43	2·76–2·96
	Do. +1·0 ,, Mo	4·32–4·40	2·85–2·93
III	*Strain W.*—0·2% G.F.2. Initial N-content, 1·00 mgm. Inc. 14 d. 30–35° C.		
	Cultures in: Basal medium (control)	1·80–1·86	0·80–0·86
	Do. +0·002 p.p.m. V	2·75–3·01	1·75–2·01
	Do. +0·005 ,, V	3·01–3·11	2·01–2·11
	Do +0·01 ,, V	3·24–(lost)	2·24
	Do. +0·1 ,, V	3·29–3·51	2·29–2·51
	Do. +1·0 ,, V	3·30–3·56	2·30–2·56
	Do. +4·6 ,, Mo	4·46–4·95	3·46–3·95
IV	*Strain W.*—0·2% G.F.2. Initial N-content, 0·89 mgm. Inc. 12 d. 35° C.		
	Cultures in: Basal medium (control)	1·42–1·43	0·53–0·54
	Do. +0·0002 p.p.m. V	1·71–1·92	0·82–1·03
	Do. +0·001 ,, V	2·37–2·61	1·48–1·72
	Do. +4·6 ,, Mo	3·00–3·16	2·11–2·16
V	*Strain W.*—0·2% G.F.2. Initial N-content, 0·78 mgm. Inc. 12 d. 30° C.		
	Cultures in: Basal medium (control)	1·57–1·64	0·79–0·86
	Do. +0·0001 p.p.m. V	1·63–1·74	0·85–0·96
	Do. +0·0002 ,, V	1·86–1·87	1·08–1·09
	Do. +0·002 ,, Mo	2·39–(lost)	1·61
	Do. +do. +0·0001 p.p.m. V	2·36–2·66	1·52–1·88
	Do. +0·004 p.p.m. Mo	2·75–2·82	1·97–2·04
	Do. +do. +0·0002 p.p.m. V	2·94–2·96	2·16–2·18
	Do. +0·100 p.p.m. Mo	3·72–3·75	2·94–2·97

With both strains a molybdenum concentration of 0·01 p.p.m., or roughly 10^{-7} molar, has an almost optimal effect, while 0·002 p.p.m. approximately doubles the fixation in the control medium, and the effect of 0·0005 p.p.m. seems detectable in strain 3. The figures are comparable to those found by Horner *et al.* (1942) for *Azotobacter*, although this seems to have a somewhat wider range of effective concentration (1 p.p.m. had optimal effect, and 0·00001–0·0001 p.p.m. had still a detectable effect in cultures 6 days old). The very small amounts of molybdenum, 0·00002–0·0001 p.p.m., show no effect at all. This suggests that the clostridia are capable of some slight nitrogen fixation in a medium devoid of available molybdenum, and that the molybdenum begins to catalyze the process when it is present in a concentration near or slightly above 0·5γ per litre of medium, or $0{\cdot}5 \times 10^{-8}$ molar. As to vanadium, the effect of 0·002 p.p.m. is still some 75% of the optimum, and 0·0002 p.p.m., or roughly $0{\cdot}4 \times 10^{-8}$ molar concentration, causes a detectable stimulation. Its active range thus seems to extend a little further than that of molybdenum, although it is less effective at optimum concentration. There is finally in Exp. No. IV a suggestion of an additive effect of 0·004 p.p.m. molybdenum and 0·0002 p.p.m. vanadium.

The Specificity of Molybdenum and Vanadium.—As mentioned in the introduction, no other elements have been found able to replace molybdenum and vanadium in *Azotobacter*. To see if the same is the case in the clostridia, as possible substitutes, we have tested some of the heavy metals of general physiological importance, namely, manganese, zinc, cobalt and copper, besides several elements of the same periodic groups as molybdenum and vanadium: chromium, tungsten, uranium, niobium, tantalum and bismuth. The experiments already discussed show that manganese is without effect; for instance, it was added to the basal medium in Exps. I, II, III, VI, X, XI and XII, Table 2. Also iron, which was always included, is obviously unable to replace molybdenum. The results of the tests with the other elements are seen in Table 5.

It seems quite clear that none of the elements tested can replace molybdenum and vanadium; several of them are even inhibitory, especially towards strain W. In the case of tantalum, the inhibition might indeed be due to an excess of potassium hydroxide in the stock solution of potassium tantalate; therefore, the test was repeated with a neutralized solution and a tantalum concentration of 0·5 p.p.m. This time growth took place, but the nitrogen fixation was not stimulated: gains in basal medium, 0·79–0·86 mgm., with 0·5 p.p.m. Ta, 0·81–0·82 mgm. (cf., Table 4, V).

Tungsten presents some special problems. On a previous occasion (Table 2, IV) it showed an appreciable stimulation, but this might be due to impurities of molybdenum, since the sodium tungstate also stimulated nitrogen fixation by *Azotobacter*, and this effect persisted after two recrystallizations (0·08–0·35 mgm. nitrogen was fixed per 25 ml. basal medium, 2·78–2·92 mgm. in medium with 10 p.p.m. sodium tungstate). A spectrographic examination of the twice recrystallized sodium tungstate, by Mr. A. C. Oertel, Waite Institute, Adelaide, showed the presence of molybdenum to the amount of some parts per million; if we assume 10 p.p.m., the addition of 10 mgm. of the salt per litre of medium would thus have provided 0·1γ of molybdenum, a quantity which, according to the data in Table 4, could hardly be expected to have any significant effect on the clostridia, although it might explain the stimulation of *Azotobacter*. A test was conducted with tungsten in varying concentration: 1·0, 0·1 and 0·01 p.p.m. The last quantity had no effect on *Azotobacter*, which in 20 ml. solution incubated 10 days at 22–25°C. (cf., Table 1, IV) showed the following gains of nitrogen:

Control medium	0·11–0·14 mgm.
Do. + 0·01 p.p.m. W	0·12–0·15 ,,

The disappearance of the stimulating effect towards strain W with increasing dilution (Table 5, IV) suggests that the effect was really due to the small amount of molybdenum impurity, although an absolutely molybdenum-free tungsten compound would be needed to supply the final answer.

TABLE 5.
Specificity of Molybdenum and Vanadium as Catalysts of Nitrogen Fixation.

Exp. No.	Strain, Medium and Incubation.	Mgm. N per Culture *a.* *b.*	Gain of N, mgm. *a.* *b.*
I	*Strain* 1.—0·4% G.F.1. Initial N-content, 0·56 mgm. Inc. 10 d. 30° C.		
	Cultures in: Basal medium (control)	1·05–1·08	0·49–0·52
	Do. +20 p.p.m. $KCr(SO_4)_2$	1·00–1·07	0·44–0·51
	Do. +10 ,, $UO_2(NO_3)_2$	1·01–1·03	0·45–0·47
	Do. +10 ,, Na_2MoO_4	2·09–2·23	1·53–1·67
II	*Strain* 1.—0·25% G.F.2. Initial N-content, 1·47 mgm. Inc. 14 d. 30° C.		
	Cultures in: Basal medium (control)	1·68–1·85	0·21–0·38
	Do. +10 p.p.m. $ZnSO_4$	1·45–1·58	(0) –0·11
	Do. +10 ,, $Co(NO_3)_2$	1·45–1·54	(0) –0·07
	Do. +10 ,, $CuSO_4$	1·29–1·35	(no growth)
	Do. +10 ,, Na_2MoO	2·25–2·30	0·78–0·83
III*	*Strain W.*—0·25% G.F.2. Initial N-content, 1·30 p.p.m. Inc. 13 d. 30° C.		
	Cultures in: Basal medium (control)	1·88–1·93	0·58–0·63
	Do. +1·0 p.p.m. Zn	1·78–1·90	0·48–0·60
	Do. +1·0 ,, Co	1·16–1·19	(no growth)
	Do. +1·0 ,, Cu	1·14–1·18	(no growth)
	Do. +1·0 ,, Cr	1·83–1·92	0·53–0·62
	Do. +1·0 ,, U	1·18–1·19	(no growth)
	Do. +1·0 ,, Nb	1·68–1·75	0·38–0·45
	Do. +1·0 , Ta	1·12–1·15	(no growth)
	Do. +1·0 ,, Bi	1·55–1·65	0·25–0·35
	Do +1·0 ,, Mo	3·45–3·53	2·15–2·23
IV	*Strain W.*—0·2% G.F.2. Initial N-content, 0·89 mgm. Inc. 12 d. 35° C.		
	Cultures in: Basal medium (control)	1·42–1·43	0·53–0·54
	Do. +1·0 p.p.m. W	2·28–2·30	1·39–1·41
	Do. +0·1 ,, W	1·45–1·47	0·56–0·58
	Do. +0·01 ,, W	1·36–1·37	0·47–0·48
	Do. +4·6 ,, Mo	3·00–3·16	2·11–2·27

* Niobium and tantalum were added as potassium niobate and tantalate, prepared by fusing the oxides with potassium hydroxide, bismuth as nitrate, the other metals in the same form as in Exps. I-II.

Upon the whole, it thus appears that the requirement of *Cl. butyricum* for molybdenum or vanadium is as specific as that of *Azotobacter*, or rather more so, since it is not all strains of clostridia that respond to vanadium.

It might here be mentioned that the stimulating influence of materials like soil or humus preparations on nitrogen fixation by clostridia, observed by Krzemieniewsky (1908) and Willis (1934), might be partly due to molybdenum or vanadium, as suggested by Bortels (1936), and partly to organic growth compounds. When Lantsch (1921) found that humus did not increase nitrogen fixation but caused a marked reduction in the length of the lag phase in clostridia, the explanation may be that these humus preparations were deficient in molybdenum but provided organic growth factors like biotin, *p*-aminobenzoic acid, etc.

The Influence of Molybdenum and Vanadium on Growth of Cl. butyricum *with Combined Nitrogen.*—A few tests were carried out to see if molybdenum and vanadium also accelerate the growth with combined nitrogen, measured by the rate of sugar fermentation. Three strains of clostridia were grown in Lampen and Peterson's (1943) medium, with addition of 0·5% G.F.1, and some calcium carbonate to prevent inhibition of the growth by accumulation of acid. Molybdenum was added as 10 p.p.m. of sodium molybdate, and vanadium as 4 p.p.m. ammonium vanadate. Test-tubes with 10 ml.

solution were inoculated with one drop of potato-medium culture and incubated in a hydrogen atmosphere at 37°C., in one test also at 30° C., and glucose was determined in duplicate or triplicate tubes at intervals mostly of 24 hours.

TABLE 6.

Rate of Fermentation of Glucose in Solution with Combined Nitrogen.

Strain.	Medium.	Per Cent. Glucose Fermented after Days.						
		1	2	3	4	6	7	8
1	−Mo	39	72	88				
	+Mo	39	73	90				
1 (30° C.) ..	−Mo	7	26	37	56		89	
	+Mo	20	25	56	56		100	
3	−Mo	15	35	47	73	96		
	+Mo	28	45	78	100	100		
5	−Mo	6	38	50	50			64
	+Mo	12	40	49	53			64
	−Mo	12	81	96				
W	+Mo	6	79	96				
	+V	36	88	100				

As seen from Table 6, molybdenum has only a small and irregular influence on the rate of fermentation, and this is most noticeable in the early stages of growth, which also appeared to start earlier in the presence of molybdenum. In the case of strain W, the same shortening of the lag phase by vanadium is quite marked, but the effect already is wearing off after 48 hours. Upon the whole, the effect of molybdenum under these conditions appears quite comparable to its influence on the growth of *Azotobacter* with ammonia-nitrogen according to Burk and Horner (1935). We might also here recall the promoting influence of molybdenum on sugar consumption by strain 3 in nitrogen-deficient medium and hydrogen atmosphere, as discussed previously.

General Conclusions.

The experimental results show quite clearly that although molybdenum and vanadium have some influence on the general metabolism of the clostridia, their effect consists pre-eminently in an acceleration of the nitrogen-fixation process, as in *Azotobacter*. It is reasonable to assume that both kinds of bacteria may possess a nitrogen-fixing enzyme ("nitrogenase") that requires molybdenum as an activator, with vanadium as a less effective substitute; a certain difference in the enzymes seems to exist, in so far as in some of the clostridia they are not activated by vanadium.

Naturally the question then arises whether the biochemical processes of nitrogen fixation are fundamentally the same in the two groups of non-symbiotic nitrogen-fixing bacteria and perhaps in all cases of biological nitrogen fixation. At the present stage this can only be a matter for conjecture, but the fact that molybdenum, and often vanadium, but apparently no other elements, catalyze nitrogen fixation in types of life so different as *Azotobacter*, *Clostridium butyricum*, the *Azotomonas insolita* of Stapp (1940), the blue-green algae (Bortels, 1940) and the association of leguminous plants and root-nodule bacteria (e.g., Jensen, 1946), certainly speaks in favour of such a unitarian concept. For verification of this hypothesis, it would, among other things, be of great value to investigate the effect of molybdenum in certain other still incompletely known or problematical agents of biological nitrogen fixation, such as the mycorrhizal fungi (*Phoma* spp.) of the Ericaceae (Ternetz, 1907; Jones and Smith, 1928), the actinomycetes from the root nodules of *Alnus* spp. (von Plotho, 1940–41), and the practically unknown root-nodule organisms of *Casuarina* spp. in which there can be little doubt about the power of nitrogen fixation (Mowry, 1933).

Summary.

Nine strains of *Clostridium butyricum* and one of *Cl. acetobutylicum* were tested for nitrogen-fixing power in a medium deficient in molybdenum but containing the necessary organic growth-compounds. All strains fixed small amounts of nitrogen in the basal medium, but the fixation was strongly increased, mostly three- to sixfold, by addition of small quantities of sodium molybdate. The yield of fixed nitrogen often exceeded three mgm. per gm. fermented glucose when adequate supplies of molybdenum were given.

A molybdenum-concentration of 0·01 part per million was nearly optimal, and the influence of 0·002 p.p.m. was still considerable. The effect of molybdenum appeared to begin at a concentration near or somewhat above $0{\cdot}5 \times 10^{-8}$ molar.

In five strains of *Cl. butyricum*, molybdenum could be replaced by vanadium, which at higher concentration mostly gave an increase of nitrogen fixation equal to one-half to two-thirds of that caused by molybdenum, but which still showed some activity at a concentration of $0{\cdot}4 \times 10^{-8}$ molar. Molybdenum could not be replaced by iron, manganese, zinc, cobalt, copper, niobium, tantalum, bismuth, chromium, uranium, or (probably) tungsten.

Molybdenum had only a relatively small or no stimulating effect on the rate of sugar fermentation with combined nitrogen (ammonia). It thus appears that molybdenum, partly replaceable by vanadium, is a specific catalyst of nitrogen fixation in *Cl. butyricum* as well as in *Azotobacter* and probably in other nitrogen-fixing forms of life.

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EXPLANATION OF PLATE VII.

Cultures of clostridia in 50 ml. of Winogradsky's solution.

1.—*Strain 1*, inc. 14 d. 30°C. (Table 2, I) ; from left : 10 p.p.m. Na_2MoO_4—Control—Sterile medium.

2.—*Strain 1*, inc. 14 d. 30°C. (Table 2, IV) ; from left : 10 p.p.m. Na_2MoO_4—1·5 p.p.m. $VOSO_4$—Control.

3.—*Strain W*, inc. 14 d. 30°C. (Table 2, XIII) ; from left : 10 p.p.m. Na_2MoO_4—4 p.p.m. NH_4VO_3—Control.

(S. Woodward-Smith photos.)